THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 61: 67–71, 1973

Vol. 61, No. 2
Printed in U.S.A.

IN VITRO STUDIES OF THE HUMAN MAMMARY GLAND: EFFECT OF HORMONES ON PROLIFERATION IN PRIMARY CELL CULTURES*

B. ALLEN FLAXMAN, M.D.

ABSTRACT

Epithelial cells from the human mammary gland were propagated up to 40 days in vitro as primary cell cultures. Large and medium-sized ducts, dissected from 4 different glands, served as a specific source of the epithelial cells. Ducts were opened, cut in small pieces, and were treated with trypsin-EDTA. The loosened epithelial cells were obtained in suspension and inoculated into Petri dishes where they attached, maintained their epithelial shape and grew as cohesive sheets of cells. The cultured cells had certain ultrastructural features in common with control uncultured cells of the ducts. In cultures from 2 mammary glands, addition of insulin or prolactin to serum-containing culture fluid did not result in any increase in the mitotic index or number of cells synthesizing DNA. Hydrocortisone, on the other hand, suppressed mitosis and DNA synthesis. This system should make possible additional studies of human mammary gland biology and pathology.

The human mammary gland is embryologically derived from epidermis (ectoderm) and as such, study of its biology falls naturally within the province of investigative dermatology. However, the functional epithelium of this organ has not been the subject of much investigation. Most studies have been concerned only with disease processes occuring in the cutaneous component, e.g., origin of the abnormal cells in Paget's disease of the nipple [1]. On the other hand, many studies of mammary gland epithelial cell behavior in nonhuman species, such as the mouse, have been performed, especially in organ and cell culture. These studies have provided useful information on hormonal effects upon the mitotic cycle, synthesis of milk proteins, and the events of carcinogenesis. Similar studies of the human mammary gland have not been performed and it is not clear whether results from animal studies are applicable to the human. A few reports have indicated that both normal and abnormal epithelial cells from the human mammary gland will grow in vitro [2–7]. These observations seem not to have been extended to the study of cell function, probably because of technical difficulties in consistently obtaining tissue where epithelial cells are present in large numbers. In the normal nonlactating human breast the relative amount of epithelium to total mass of the organ is quite small. Consequently, explants or cell suspensions made from randomly chosen pieces of tissue are most likely to yield fibroblasts. Recently, it has been reported that the readily identifiable ducts of the nipple and subjacent tissue which can be dissected from the human mammary gland can be used as a highly specific source of epithelial cells [8]. The present report describes how large numbers of cells can be grown in vitro in order to study the effect of hormones on DNA synthesis and mitosis. The ultimate purpose of this work is to make possible studies of normal human mammary gland epithelial cell behavior in vitro that will provide a baseline for analysis of human mammary gland carcinogenesis, an approach heretofore not possible because of inadequate methods of culture.

MATERIALS AND METHODS

Primary cell cultures were made from specimens of four different mammary glands. The nipple and subjacent mammary tissue were obtained from postmenopausal females following mastectomy because of a carcinoma. Mastectomy material was used in the present study because it was available for culture almost immediately. The experimental material was quite distant from the tumor and was grossly free of disease. A further check was made by random histologic sampling of isolated ducts. Although it is not yet possible to define the "normal" state of the human mammary gland, previous studies showed that in explant cultures of ducts from uninvolved areas of mammary glands with carcinomas, epithelial growth characteristics were the same as for material obtained from "normal" glands at autopsy [8].

By careful dissection, the mammary ducts could be visualized in longitudinal and transverse section with the aid of a dissecting microscope. The epithelium had a distinct yellow tinge that aided identification. The quantity of duct material that could be obtained was variable, since some mammary glands had an extensive system whereas in others the system was only minimally developed. Isolated ducts, comprising the epithelium and surrounding connective tissue, were placed in Gey's

Manuscript received February 23, 1973; in revised form April 11, 1973; accepted for publication April 16, 1973.

This work was supported by grants from The National Cancer Institute, No. 1 PO 1 CA 11536, and The National Institute of Arthritis and Metabolic Diseases, No. 1 PO 1 AM 15515.

Prolactin was a gift of the National Pituitary Agency, National Institutes of Health.

* From the Department of Dermatology, The Skin and Cancer Hospital of Philadelphia, Temple University Health Sciences Center, Philadelphia, Pennsylvania, 19140.

balanced salt solution, opened longitudinally and cut into fragments 3–4 mm square (20–50 fragments, depending on the quantity of duct that could be dissected). Fragments were incubated at 37° C for 20–30 min in a 0.25% trypsin-EDTA mixture (Grand Island Biological Co.) after which they were placed in culture fluid containing 10% calf serum. With the aid of fine forceps the epithelium was readily stripped or scraped away from the underlying connective tissue. The pooled epithelial fragments from each mammary gland were spun down at low speed in a centrifuge, the supernatant fraction removed, and the fragments resuspended in a small volume of culture fluid. With gentle agitation through a fine-bore pipette, the fragments were broken up even further. The final suspensions consisted of individual cells and clumps of cells that were resistant to further dispersion. Because of clumping, no attempt was made at cell counting. Each cell suspension was further diluted in culture fluid and identical aliquots immediately pipetted into plastic Petri dishes, each containing a coverslip. The extent of dilution was such that the number of cells per ml was sufficient to give the culture fluid a cloudy appearance. All cultures were grown in Eagle's Minimal Essential Medium with 10% fetal calf serum, and were incubated at 37° C in a high humidity incubator in an atmosphere of 5% CO_2 in air.

For electron microscopy, cultures or pieces of uncultured duct were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hr at room temperature, postfixed for 1 hr in a 1:2 mixture of osmium and collidine [9], dehydrated through alcohol and propylene oxide, and embedded in araldite.

In experiments using some of the cells from 2 of the 4 mammary glands, hormones, alone or in combination, were added to the culture fluid and were present from the outset to the termination of each experiment—a total of 10 days. Hormone concentrations were as follows: insulin 3.63 μgm/ml; hydrocortisone 5 μgm/ml; bovine prolactin 5 μgm/ml; a combination of insulin, hydrocortisone, and prolactin each in the above concentrations. Culture fluid was renewed every 3 days. On the 10th day, tritiated thymidine (6.7 Ci/mM) was added at a concentration of 2 μc/ml for 1 hr. Cultures were fixed in Bouin's fluid, dipped in Kodak NTB-2 emulsion, and exposed for 2 weeks at 4° C. After development, they were stained with hematoxylin. Both the labeling and mitotic indices were determined. The figures represent counts made on 3000 or more cells per coverslip (n = 3 coverslips for each test system in two separate experiments).

RESULTS

Morphologic observations. Successful growth was obtained for up to 40 days in cell cultures from all four mammary gland specimens. Most cells and cell clumps attached to the coverslip by 24 hr. By 2 days, small cell islands had formed (Fig. 1) that grew progressively in area (Fig. 2). Many of the islands coalesced to form large sheets nearly covering the surface of the Petri dish (Fig. 3). The cells were mainly epithelial in shape, were closely adherent, and grew as an imperfect monolayer with a small amount of cytoplasmic but no nuclear overlap. Fibroblasts were not seen. Attempts at subculturing healthy-appearing confluent cultures were all unsuccessful. Following enzymatic separation from the Petri dish, the cells were able to attach to the bottom of new dishes, but mitotic behavior remained low or absent over a 2-week observation period.

Ultrastructural examination showed the cells to

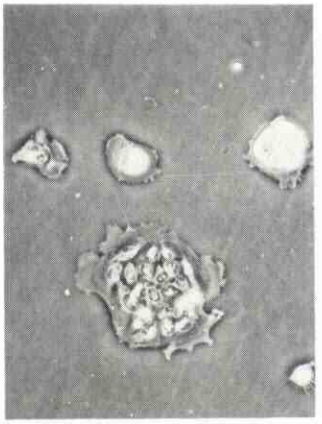

FIG. 1. Phase contrast light micrograph of small islands of epithelial cells after 2 days in vitro. × 125.

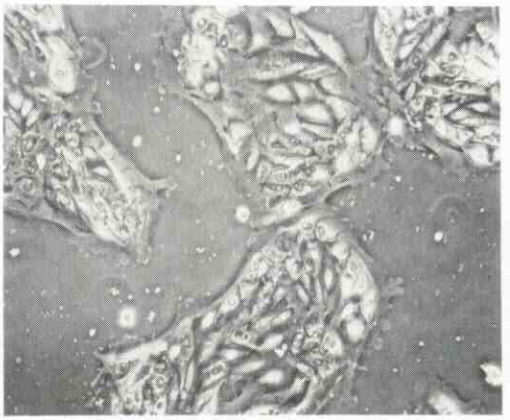

FIG. 2. Same area as in Fig. 1 showing enlargement of cell islands after 6 days in vitro. × 125.

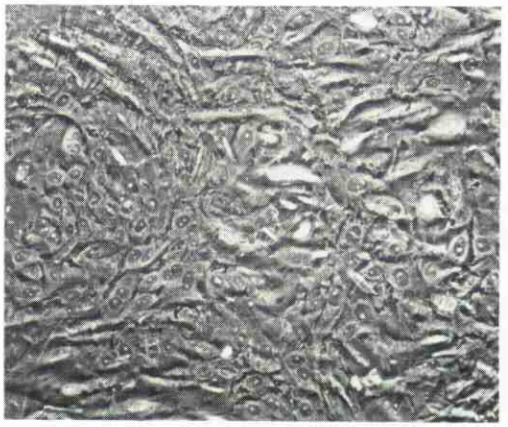

FIG. 3. Same area as in Fig. 2 after 10 days in vitro. × 125.

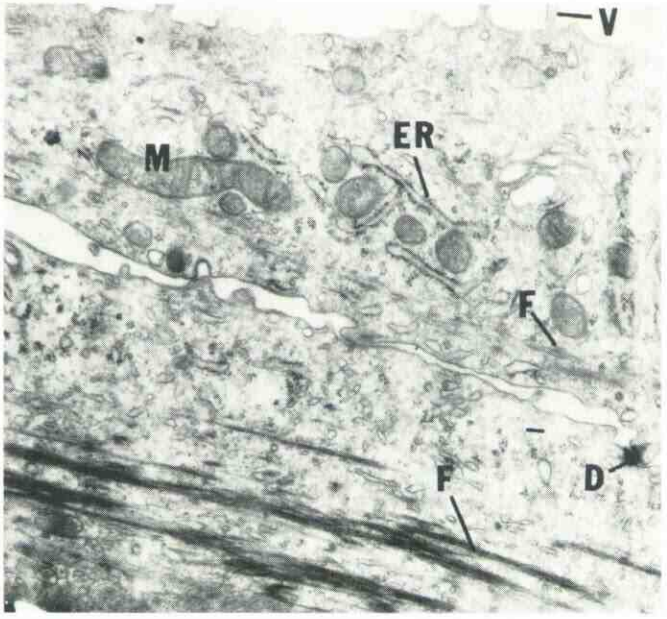

FIG. 4. Electron micrograph of epithelial cells at region of cytoplasmic overlap. Note villi (V), mitochondria (M), profiles of endoplasmic reticulum (ER), desmosomes (D), bundles of large filaments (F), more numerous and of greater electron density in the lower cell. × 22,500.

be quite flat with some cytoplasmic overlap (Fig. 4). Cells were linked by small desmosomes. Villi were sometimes present on the upper cell surface. The cytoplasm contained numerous mitochondria and profiles of rough endoplasmic reticulum. Filaments, 50–70 Å in diameter, resembling tonofilaments, were seen in longitudinal or cross section in most cells. These filaments were more numerous and of greater density in some cells than in others. No keratinized cells were found. Electron micrographs of uncultured ducts revealed that cells having similar cytoplasmic features were present in the epithelium.

Effect of hormones on proliferation. Cells incubated with tritiated thymidine showed incorporation of label into nuclei (Fig. 5). From the Table it is clear that in the presence of serum the epithelial cells in primary cultures proliferated readily and that about 20 percent were in S phase at the end of 10 days in vitro. Neither insulin nor prolactin significantly increased DNA synthesis or mitosis. In contrast, hydrocortisone alone or in combination with insulin and prolactin caused a marked depression of both DNA synthesis and mitosis to about 50 percent of the control value. Addition of insulin and prolactin to subcultured cells did not promote proliferation.

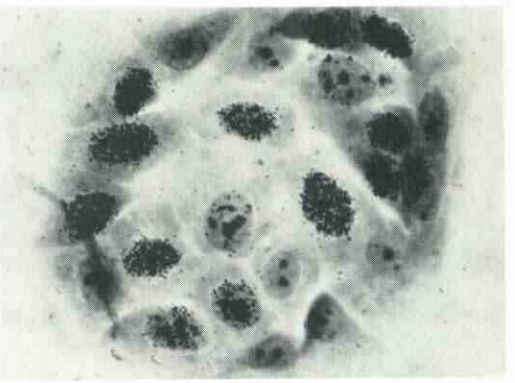

FIG. 5. Autoradiograph of small island of epithelial cells after exposure to tritiated thymidine for 1 hr. A high percentage of nuclei are labeled. × 475.

DISCUSSION

The opportunity to study the human mammary gland in the experimental situation has not been exploited by those involved in cutaneous biology. This is surprising since the mammary gland is an epidermal derivative whose hormone responsiveness bears at least a superficial resemblance to similar behavior of hair roots and sebaceous and apocrine glands. Whereas cancer of these latter appendages is rare, carcinoma of the mammary gland is among the most common of serious cancers in women. Thus, comparative studies of epithelial behavior may be important in explaining the incidence and behavior of mammary gland cancer.

In the present study there can be little doubt of the epithelial origin of the cultured mammary gland cells whose shape and growth characteristics were similar to those of other types of epithelial cells in vitro. Ultrastructural features were similar to those of cells in the ducts themselves as shown

TABLE

Effect of hormones on DNA synthesis and mitosis in primary cell cultures of human mammary gland epithelium

Each variable was studied in 3 cultures from 2 different mammary glands (total of 6 cultures). Counts are based on 3,000 or more cells from each dish (total of 18,000 or more cells per variable).

	Labeling Index (per 1000 cells)	P	Mitotic Index (per 1000 cells)	P
Control	206 ± 23	—	22.6 ± 2.3	—
Insulin (I)	183 ± 30	N.S.	24.6 ± 2.08	N.S.
Prolactin (P)	203 ± 25	N.S.	24 ± 4.5	N.S.
Hydrocortisone (H)	110 ± 20	<.01	10.3 ± .57	<.005
I, H, P	113 ± 25	<.015	9.6 ± 1.5	<.005

N.S. = Not Significant.

both in the present study and the reports of others [10–16]. Cell connections by well-formed desmosomes as well as the presence of tonofilament-like structures, while not absolutely diagnostic of epithelial cells, tend to indicate an origin from epithelium. Failure to find keratinized cells probably indicates that during development, pathways of keratin synthesis have been turned off. Squamous metaplasia and pearl formation does occur in some breast carcinomas, indicating that the potential for keratinization continues to be present [17].

Although hormones appear to play a role in human mammary gland development and proliferation in vivo, there is little experimental analysis of their specific effects. On the other hand, there have been many studies of the effect of hormones on proliferation and function in vitro of cells from nonhuman mammary glands. Serum has been shown to stimulate proliferation of mouse mammary gland epithelial cells in vitro [18]. The effect is not related to its content of insulin or prolactin, both of which stimulate proliferation in serum-free media [19–24]. It should be stressed that the hormonal studies in the present investigation are limited in nature but that the concentrations used are similar to those that have been found to be effective for nonhuman mammary glands. The failure of insulin and prolactin to augment proliferative activity in the present system, therefore, may mean that serum alone elicits a maximum response. Hydrocortisone, which depressed DNA synthesis and mitosis in our cultures, is required for synthesis of specific milk proteins in organ culture of mouse mammary gland [21, 22, 25], but a concomitant depressant effect on DNA synthesis in this latter system has not been reported. In the present study, failure to obtain continued proliferation following subculturing, even in the presence of serum or hormones, remains unexplained. Conceivably, treatment with enzymes damages the cells significantly, although their ability to attach to a new substratum would indicate only minor, if any, damage.

The results of the present study show that epithelial cells from the human mammary gland can be obtained in a pure form as primary cell cultures. Although attempts to subculture the cells have not yet been successful, the method of primary cell culture should permit a variety of further studies of function and carcinogenesis not previously possible.

REFERENCES

1. Sagebiel RW: Ultrastructural observations on epidermal cells in Paget's disease of the breast. Am J Pathol 57:49–61, 1969
2. Coman DR: Human neoplasms in tissue culture. Cancer Res 2:618–625, 1942.
3. Foley JF, Aftonomos BT: Growth of human breast neoplasms in cell culture. J Natl Cancer Inst 34:217–229, 1965
4. Lasfargues EY, Ozello L: Cultivation of human breast carcinomas. J Natl Cancer Inst 21:1131–1147, 1958
5. Orr MF, McSwain B: Tissue culture of human breast carcinoma and normal breast epithelium. Tex Rep Biol Med 12:916–920, 1954
6. Orr MF, McSwain B: Tissue culture of human breast carcinoma. Am J Pathol 31:125–141, 1955.
7. Whitescarver J, Recher L, Sykes JA, Briggs, L: Problems involved in culturing human breast tissue. Tex Rep Biol Med 26:613–628, 1968
8. Flaxman BA, Van Scott EJ: Growth of normal human mammary gland epithelium in vitro. Cancer Res 32:2407–2412, 1972
9. Bennett HS, Luft JH: S-collidine as a basis for buffering fixatives. J Biophys Biochem Cytol 6:113–114, 1959.
10. Carter D, Yardley JH, Shelly WM: Lobular carcinoma of the breast. An ultrastructural comparison with certain duct carcinomas and benign lesions. John Hopkins Med J 125:25–43, 1969.
11. Murad TM, Scarpelli DG: The ultrastructure of medullary and scirrhous mammary duct carcinoma. Am J Pathol 50:335–360, 1967.
12. Murad TM, Van Haan E: Ultrastructure of myo-epithelial cells in human mammary gland tumors. Cancer 21:1137–1149, 1968
13. Ozello L: Ultrastructure of the human mammary gland, Pathology Annual. Sixth Edition. Edited by SC Sommers. New York, Appleton–Century–Crofts, 1971, pp 1–59
14. Tannenbaum M, Weiss M, Marx AJ: Ultrastructure of the human mammary ductule. Cancer 23:958–978, 1969
15. Toker C: Observations on the ultrastructure of a mammary ductule. J Ultrastruct Res 21:9–25, 1967
16. Waugh D, VanderHoeven E: Fine structure of the human adult female breast. Lab Invest 11:220–228, 1962
17. Haagensen CD: Diseases of the Breast. Philadelphia, WB Saunders Company, 1971
18. Majumder GC, Turkington RW: Stimulation of mammary epithelial cell proliferation *in vitro*. Endocrinology 88:1506–1510, 1971
19. Dilley WG: Morphogenic and mitogenic effects of prolactin on rat mammary gland *in vitro*. Endocrinology 88:514–517, 1971
20. Elias JJ: Effect of insulin and cortisol on organ cultures of adult mouse mammary gland. Proc Soc Exp Biol Med 101:500–502, 1959.
21. Jeurgens WC, Stockdale FE, Topper YJ, Elias, JJ: Hormone-dependent differentiation of mammary gland *in vitro*. Proc Natl Acad Sci USA 54:629–634, 1965

22. Lockwood DH, Stockdale FE, Topper YJ: Hormone-dependent differentiation of mammary gland: sequence of action of hormones in relation to cell cycle. Science 156:945–946, 1967
23. Rivera EM, Bern HA: Influence of insulin on maintenance and secretory stimulation of mouse mammary tissues by hormones in organ culture. Endocrinology 69:340–352, 1961
24. Turkington RW: Hormone-induced synthesis of DNA by mammary gland *in vitro*. Endocrinology 82:540–546, 1968
25. Mills ES, Topper YJ: Some ultrastructural effects of insulin, hydrocortisone and prolactin on mammary gland explants. J Cell Biol 44:310–328, 1970